

SEASONAL CHANGES IN COLD HARDINESS OF THE INVASIVE FRESHWATER
APPLE SNAIL, *POMACEA CANALICULATA* (LAMARCK)
(GASTROPODA: AMPULLARIIDAE)

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ABSTRACT

We investigated the cold hardiness of a South American freshwater apple snail, *Pomacea canaliculata*, which began to invade Japanese paddy fields in the early 1980s. *Pomacea canaliculata* exhibited apparent seasonal fluctuation in its cold hardiness. Snails collected from submerged paddy fields in summer were less tolerant to cold, and none survived exposure to 0°C for five days. With decreasing temperature, together with drainage of its habitat in autumn, *P. canaliculata* developed cold hardiness, attaining almost 100% survivorship after exposure to 0°C for five days in December. The snails resting in drained fields were approximately nine times more cold tolerant than those collected from submerged fields, based on the time to 50% mortality at 0°C. Snails overwintering in aquatic conditions also acquired cold hardiness.

Key words: apple snail, cold hardiness, invasion, overwintering, rice field, *Pomacea canaliculata*.

INTRODUCTION

The apple snail *Pomacea canaliculata* (Lamarck, 1819), often referred to as the golden apple snail in Southeast Asia, was introduced into Japan and many other countries in Southeast and East Asia in the early 1980s (Mochida, 1991; Litsinger & Estano, 1993). This freshwater snail, originating from South America, was initially introduced as a source of human food. However, commercial markets failed, and discarded or escaped snails invaded rice ecosystems, becoming a serious pest of rice in many countries (Naylor, 1996; Wada, 2004). *Pomacea canaliculata* is naturally distributed in a wide area from the Pantanal, Brazil, to 38°S in Argentina, mostly inhabiting shallow lakes, ponds, swamps and the catchment areas of rivers (Halwart, 1984; Martin et al., 2001). The origins of Asian *P. canaliculata* are unknown, but may include many sources.

In Japan, *P. canaliculata* mostly inhabits paddy fields and irrigation canals. In paddy fields, *P. canaliculata* shows explosive population growth every year. In Kumamoto, south-

ern Japan, normal season rice is planted in June. Before rice planting, drained paddy fields are irrigated for harrowing a few days before the seedlings are planted. This irrigation breaks the dormancy of snails that have overwintered there. The overwintered population of the snails comprises a majority of juveniles of intermediate size (shell height approximately 7–18 mm) (Watanabe et al., 2000; Wada et al., 1999) that were born in the previous crop season, and very small number of adult snails, most of which seem to have survived through the two previous crop seasons. The total snail densities are usually not high, for example $2.8 \pm 1.1 \text{ m}^{-2}$ (mean $\pm$ S.E.) in paddy fields of northern Kyushu in 2000 (Wada et al., 2004). The adult snails soon begin sexual activity, and females lay egg masses in the fields. The adults grow very slowly, probably because of heavy investment in reproductive activity. On the other hand, the juveniles grow very rapidly, becoming adults in a month or so after paddy irrigation. Thus, the two types of snails present at irrigation overlap in size, and cannot be separated on that basis in July. Almost simultaneously, juveniles of the new gen-

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eration appear in paddy fields. Thus, again, two types of snails in terms of size constitute the snail population in summer (Syobu, 1996). Snails tend to stop growing after August, due to a paucity of food (Tanaka et al., 1999). However, population densities gradually increase due to continuous appearance of the new generation during the irrigation period. By early autumn, the population densities often reach a few hundred/m² (the majority being juveniles smaller than 10 mm), which is sometimes more than 100-fold the initial overwintered population (Wada et al., 2004). This population explosion is attributed to the lack of natural enemies in paddy fields (Yusa et al., 2006). Most juveniles of the new generation do not reach adulthood until drainage of the paddy fields in late September before the rice harvest. When farmers stop irrigation, many or most of the snails bury themselves in the soil and enter dormancy prior to overwintering. However, many snails fail to bury themselves, and instead overwinter under straw scattered on the fields at harvest time in mid October. Mortality of adult snails in winter is higher than that of juveniles of intermediate size, because intermediate-sized snails are more tolerant to the cold (Syobu et al., 2001; Oya et al., 1987), and also the tillage sometimes carried out by farmers during the fallow season kills large snails (Takahashi et al., 2002). Therefore, it seems difficult for *P. canaliculata* individuals to survive more than three crop seasons in paddy fields. In comparison with paddy snails, the ecology of *P. canaliculata* inhabiting irrigation canals has been scarcely studied, probably because of the difficulty in tracing populations. Very large snails are often found in canals, and therefore some snails in this environment appear to have a much longer life span than paddy snails.

Winter mortality of this invasive snail apparently constrains its distribution and reproduction in Japan. The northernmost known population of the snail in Ibaraki, central Japan, cannot overwinter in paddy fields, which are recolonized every year by snails that have overwintered in a limited area in canals (Ito, 2002). Even in Kyushu, southern Japan, the snail is distributed mainly in lowland areas and cannot invade paddy fields in mountain areas, where the temperature decreases considerably in winter. Winter mortality of the hibernating snails in paddy fields reportedly varies. In Kyushu, estimated mortality has been reported to be 65–95% in Chikugo (Oya et al., 1987) and 85% in Kumamoto (Watanabe et al.,

2000), and 18–67% mortality has also been reported for snails hibernating under rice straw in Saga (Syobu et al., 2001). In Shizuoka, which has a generally warmer winter, mortality in winter has been estimated to be less than 40% (Ozawa & Makino, 1988). However, these figures probably underestimate mortality, because small snails, which usually comprise the majority of the snails in autumn paddy fields and are less tolerant of cold weather (Syobu et al., 2001; Oya et al., 1987), were often not included in the data. In fact, Wada et al. (2004), who carefully investigated population densities including very small snails, reported that the difference in densities between in early summer and autumn fields was 60- to 940-fold. Thus, the populations of *P. canaliculata* in Japan collapse during winter, and this is a key constraint on population growth of the invasive snail in its new habitat in temperate Japan.

A few studies have experimentally examined the cold hardiness of *P. canaliculata* in Japan, and generally these have demonstrated that the snail is not tolerant of cold temperatures. For example, laboratory-reared progeny of snails collected in four locations in Japan and the Philippines mostly died within three days at 0°C, irrespective of their origin (Sugiura & Wada, 1999). Ohgami (1986) also reported that most snails died after several days at 2°C. However, according to Oya et al. (1987), snails collected in winter survived more than ten days at 0°C. Thus, it seems that there is some degree of discord among previous studies with respect to the cold hardiness of *P. canaliculata*.

In this study, we attempted to clarify the reason for this discord by sampling the snails periodically from fields. There is a possibility that *P. canaliculata* has evolved to enhance cold hardiness in Japanese paddy fields, because it has been selected by cold Japanese winters for more than 25 years. In addition, it is important for us to know winter mortality of the snails and its mechanism so as to develop a new management suppressing the population of pest snails during winter.

MATERIALS AND METHODS

Snails Used

The snails used were collected from paddy fields in Kikuchi, Kumamoto Prefecture (32°57'N, 130°45'E), except as indicated below. We collected active snails from sub-

merged paddy fields during summer and snails resting under straw from drained fields in autumn and winter. When we collected snails from submerged fields, we were able to discriminate live snails from dead ones by the presence of an operculum. Under aquatic conditions, the operculum of a dead snail soon disappears from the shell. However, under dry conditions, since a dead snail retains its operculum for many days, we were unable to discriminate live snails when collecting in drained paddy fields. Therefore, we had to examine snail mortality using the method described below, while simultaneously testing for cold hardiness. When the initial mortality was not zero, we applied Abbott's correction (Abbott, 1925) to the results of the cold hardiness tests.

Differences in Cold Hardiness According to Size

Since few reports (Syobu et al., 2001; Oya et al., 1987; Watanabe et al., 2000) have documented differences in cold hardiness of *P. canaliculata* according to size, we first evaluated such differences in detail. We collected snails on September 15, 2005, from a few submerged paddy fields. The snails were washed with tap water and then left at room temperature until dry. Then, the snails were divided into five classes according to size and sex: very small juveniles with shell heights of 5–7.5 mm, small juveniles of 7.6–12.5 mm, juveniles of 12.6–20.0 mm, adult males (shell heights exceeding 23.0 mm) with peculiarly shaped opercula and apertures (Cazzaniga, 1990), and adult females with shell heights larger than approximately 25.0 mm, since females mature at this size (Tanaka et al., 1999). Thirty snails of each size class (25 snails for adults) wrapped with wet paper were confined in a plastic container (11 cm x 9 cm, 4 cm in depth for juveniles, and 19 cm x 12 cm, 6 cm in depth for adults) and kept in an incubator for two days at temperatures of 3°C, 0°C, -3°C and -5°C ($\pm 1.0^\circ\text{C}$). Two replicates were carried out. After incubation, we discriminated live snails from dead ones as described below.

Seasonal Variation

To examine seasonal fluctuation in the cold hardiness of *P. canaliculata*, we periodically collected juveniles from a paddy field from June 2005 to March 2006. The shell heights of the snails were fixed from 7.5 to 17.5 mm

(adult snails were collected only on December 7). On some occasions, we randomly selected 50 to 100 snails from the collected samples and measured their shell heights with calipers to monitor snail size. The first sampling occasion was on June 21, about a week after initial irrigation of the field for harrowing. Thus, the samples were overwintered snails that had broken their dormancy. From the second sampling occasion onwards, the snails sampled were the progeny (first generation) of the overwintered snails (see Introduction). Snails collected when the paddy field was drained (samples of October and thereafter) were checked for mortality, while cold treatment tests were simultaneously conducted. For cold treatment, 25 to 60 snails were wrapped with wet paper and kept in a plastic container (11 cm x 9 cm, 4 cm). They were then incubated for two days at 3°C, 0°C, -3°C, and -5°C, and for five days at 0°C (two replicates). In January 2006, survival of field snails declined considerably, and therefore it became difficult to carry out the cold treatment tests thereafter.

On March 8 and 15, we sampled the paddy soil containing hibernating snails to estimate roughly the mortalities and densities of the snails that had successfully buried themselves. First, we sampled a 0.25 m² area of soil (0.5 m x 0.5 m, 0.1 m depth) at 12 locations selected approximately randomly in the field after removing straw and snails on the soil surface. We brought the soil to the laboratory and carefully removed the snails from the soil by washing the soil away through a fine-mesh metal bowl. We then measured the size of the snails and checked whether they were alive. Similarly, we collected all snails resting under straw from a 0.25 m² quadrat at 16 locations in the field and estimated the mortality and density of the snails. We conducted further sampling of snails under the straw in additional areas of the field to increase the sample size. The March 8 and 15 data were combined, because the area of soil sampled on each occasion was too small.

Temperatures under the rice straw and on the soil surface without straw were monitored with data loggers every hour from December 8 to March 14. We also used data measured by the Japanese Meteorological Agency at the AMeDAS (Automated Meteorological Data Acquisition System) point at Kikuchi, 3 km from the survey field, as a substitute for air temperatures at 1.2 m above the soil surface.

Survivorship at 0°C

We compared survivorship at 0°C of snails collected in summer and those collected in winter. Hibernating snails were collected from a drained paddy field on January 8 and 18, 2005. Summer snails were collected from submerged paddy fields on July 21 and August 8, 2006. The shell heights of the snails collected ranged from 7.5 to 12.5 mm. Thirtyfour to 60 snails in each treatment period, wrapped with wet paper were kept in a container (11 cm x 9 cm, 4 cm), and incubated at 0°C for various periods (no replicate). After incubation they were subjected to the mortality test.

Cold Hardiness of Overwintering Snails under Aquatic Conditions

We collected overwintering snails from a concrete water tank placed outdoors in Kyushu National Agricultural Research Center on February 18, 2005, to examine the cold hardiness of overwintering snails under aquatic conditions. The snails were a stock culture that had originated from paddy fields in Kikuchi. The shell heights of snails collected ranged from 7.5 to 12.5 mm. Forty snails in each treatment were wrapped with wet paper and kept in a container (11 cm x 9 cm, 4 cm) (two replicates). They were then incubated at 0°C for five days and ten days and subjected to the mortality test.

Mortality Test

For the snails collected from drained fields and the snails that had been subject to cold tolerance treatments, we recorded the proportions of individuals that survived (mortality test). The snails were immersed in tap water in a glass container and left at room temperature (20°C to 28°C) for approximately one day. If the snails were alive, they opened their opercula and extruded their soft parts. Therefore, we considered snails that extruded the foot and/or tentacles from the shell within a day as alive. Some snails half-opened their opercula and responded by closing them when probed with a forceps. We regarded such snails as dead because most exhibiting this type of response finally died.

Statistical Analysis

In this study, the experiments were conducted basically in a two-replicate design. The proportions of snails that survived after cold treatment attained generally similar values

between replicates, except for the treatments at -3°C. Therefore, we combined the data from the two replicates, calculating the net proportions of survivorships for each treatment. Then, analysis of multiple comparisons of proportions was carried out according to the method of Zar (1996).

To obtain the LT_{50} (time until 50% of the snails died) at 0°C, we used the regression of the proportions of surviving snails against treatment time (days). The percentage data were transformed into prohibits before regression calculation.

RESULTS

Differences in Cold Hardiness According to Size

Juveniles of intermediate size were more tolerant of cold temperature than “very small juveniles” and adults (Fig. 1). This was apparent when snails were kept at 0°C for two days. More than half of the “small juveniles” and “juveniles” survived, while the survivorships were about 10% for “very small juveniles” and adults, the differences being significant. At -3°C and lower temperatures, no snails except for a juvenile survived for two days. No difference in cold hardiness was found between the sexes. However, when considering survivorships at 3°C within each sex, bigger males were more cold tolerant than smaller ones, that is, the average shell height of viable males (mean $\pm$ S.D. 30.5 ± 3.3 mm) was significant larger (t -test, $P < 0.001$) than that of dead snails (25.9 ± 1.8 mm). This was not the case for females (alive, 31.7 ± 2.5 mm; dead, 31.7 ± 3.2 mm, t -test; $P > 0.9$).

Survivorship of Field-Collected Snails and Changes in Cold Hardiness

A majority of the juveniles collected up to December 2005 under straw in the field were alive (Table 1), but by January 2006, only 27% of juveniles under the straw were alive. By mid March, only 1.4% were alive. In mid March, we sampled snails hibernating in the soil as well as those under straw. In the soil, 7.8% of the juveniles (7.5–17.5 mm) were alive, which was significantly higher than for juveniles under straw (Fisher's exact probability test, $0.001 < p < 0.01$). Juveniles smaller than 7.5 mm were all dead, both in the soil and under straw. The roughly estimated densities of live and dead very small juveniles (< 7.5) and juveniles

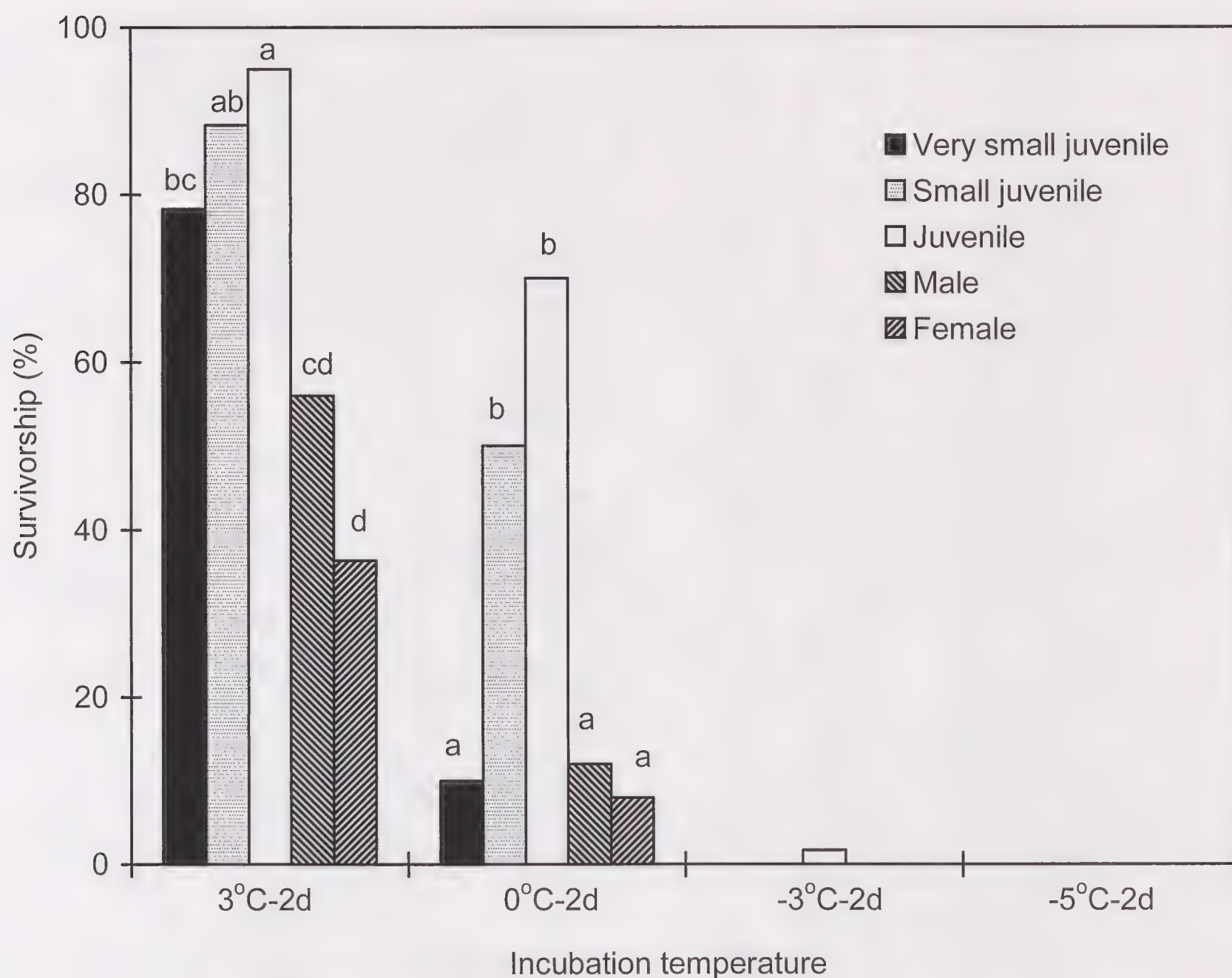


FIG. 1. Survivorships of *Pomacea canaliculata* juveniles of different sizes exposed for two days at various low temperatures. Very small juveniles, 5.0–7.5 mm; Small juveniles, 7.6–12.5 mm; Juveniles, 12.6–20.0 mm in shell height. Male: male adult, Female: female adult.

(7.5–17.5 mm) in the soil were 26.0/m² and 34.5/m², respectively, about twice those of snails under straw (11.0 and 17.8/m², respectively), although the densities of very small snails might have been underestimated because of the difficulty in finding them. Very few snails larger than 17.5 mm were found in either the soil or under straw.

In the cold treatments, most juveniles survived two days at 3°C, irrespective of sampling season (Table 1). At 0°C for two days, the proportions of surviving juveniles varied. The lowest survival rate (20%) was recorded for progeny of overwintered snails collected on August 6, during the hottest season of the year. Overwintered snails collected in June and their progeny collected in September, both from submerged paddy fields, showed comparatively low survival rates. On the other hand, almost all snails collected in autumn and winter from the drained field survived this cold temperature treatment, implying a seasonal fluctuation in the cold hardiness of *P. canaliculata*. At -3°C for two days, considerable numbers of juveniles collected on October 13 and December 7 survived. However, these

data varied widely among replicates (containers), despite the identical incubation conditions. For example, the survivorships of two replicates were 4% and 76% (average 40%) in the samples taken on October 13, and 2.5% and 100% (average before Abbott's correction 51%) in the samples taken on December 7. Because -3°C is perhaps close to the supercooling point of the snail, the great variation among the replicates may have been because of accidental inoculative freezing.

Seasonal fluctuation in cold hardiness was conspicuous when snails were kept at 0°C for five days. No juveniles collected in summer survived, whereas the survivorships increased from October and went up to almost 100% in December. The adult snails collected on December 7 also showed a high survival of 80% (24 out of 30 sampled) when exposed to 0°C for five days. Thus, seasonal fluctuation in cold hardiness was apparent in *P. canaliculata* inhabiting paddy fields in Kumamoto. In January, a corrected value of 37% survivorship after exposure to 0°C for five days was recorded. However, this value was unreliable because the initial survivorship (27%) of the samples

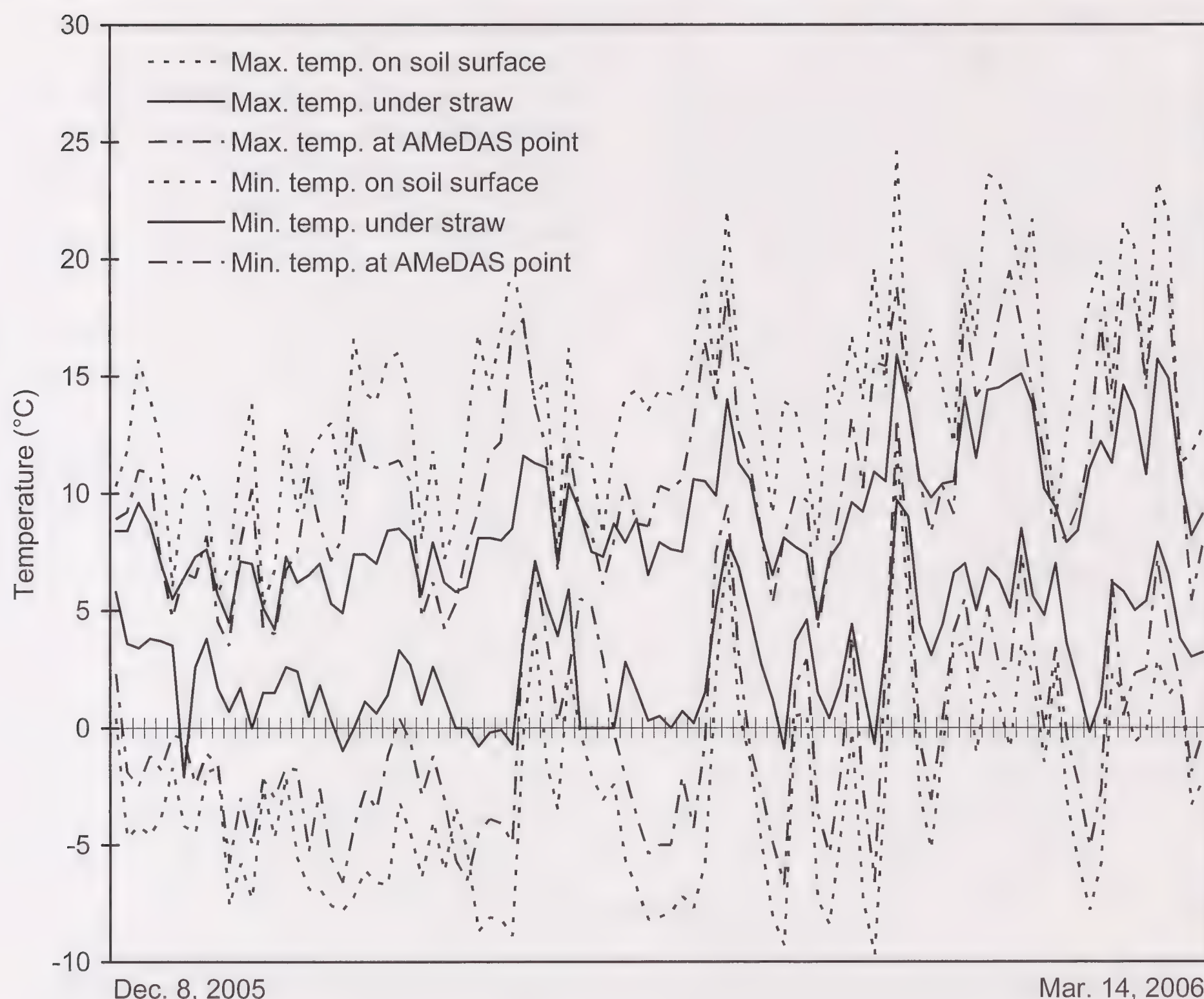


FIG. 2. Daily maximum and minimum temperatures measured on the soil surface, under rice straw, and at the nearest AMeDAS (Kikuchi) point from the survey field.

was too low for Abbott's correction. After this, we discontinued the cold treatment test, because the mortality of field snails was too high.

Cold weather was prevalent in Kyushu in the winter of 2005–2006. Nevertheless, the environment under the rice straw was well buffered thermally, and the temperature scarcely fell below zero (Fig. 2). Daily minimum temperatures under the straw were, on average, 5.0°C and 1.6°C higher than those on the soil surface and at the AMeDAS point, respectively. Similarly, daily maximum temperatures under the straw were 6.0°C and 3.3°C lower than those at the other two points, respectively.

Survivorship at 0°C

Many snails collected in January from the drained field survived more than 10 days with exposure to 0°C (Fig. 3). The LT_{50} value calculated from the regression ($Y = -0.21X + 2.46$, $r^2 = 0.89$, Y : probit of percentage mortality, X : days after 0°C incubation) was 11.9 days. On

the other hand, all snails collected in July and August from the submerged fields died within three days at 0°C, having a much shorter LT_{50} of 1.3 days ($Y = -2.07X + 2.69$, $r^2 = 0.89$). Based on the LT_{50} values, winter snails were about 9.2 times more tolerant to the cold temperature than summer snails.

Cold Hardiness of Overwintering Snails under Aquatic Conditions

The snails overwintering outdoors under aquatic conditions showed survivorships at 0°C of 67.5% (± 7.5 ; S.E.) and 22.5% (± 7.5) for five and 10 days, respectively. The values for five days representing the extent of cold hardiness were almost comparable to those for the snails collected in winter from the drained paddy field, and were higher than those for the snails collected in summer from the submerged fields. Thus, the snails overwintering under aquatic conditions also developed cold hardiness.

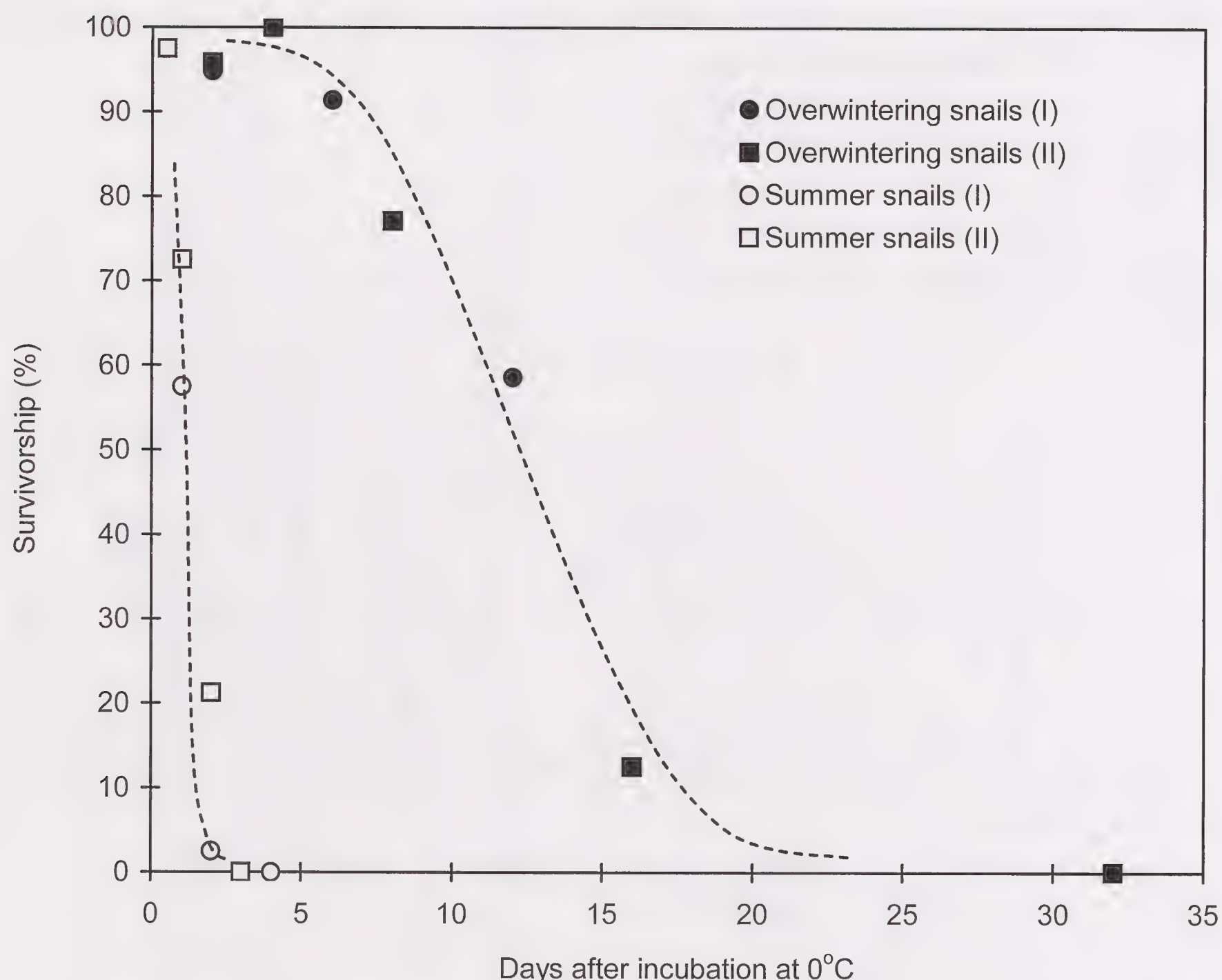


FIG. 3. Differences in survivorships after exposure to 0°C between juveniles of *P. canaliculata* collected in winter and in summer. The overwintering snails were collected from a drained paddy field on January 8 (I) and on January 18, 2005 (II). The summer snails were collected from submerged paddy fields on July 21 (I) and on August 8, 2006 (II).

DISCUSSION

We observed apparent seasonal fluctuation in the cold hardiness of *P. canaliculata*. Summer snails were less tolerant to cold even at temperatures above zero. With decreasing temperature, together with drainage of its habitat, *P. canaliculata* developed cold hardiness. Such environmental conditions enhanced the degree of cold hardiness (Matsukura & Wada, submitted). Snails overwintering under aquatic conditions also acquired cold hardiness in winter.

The strategies adopted by ectotherms for overcoming winter cold have been frequently reviewed (for example, Storey & Storey, 1997; Ansart & Vernon, 2003). The three main strategies are: moving to a favorable habitat (behavioral freezing avoidance), avoidance of freezing by extensive supercooling (physiological freezing avoidance), and survival despite partial freezing (freezing tolerance). However, there is limited information on cold hardiness

in snails. In intertidal species, freezing tolerance is the major strategy, and numerous examples of snails exhibiting seasonal fluctuations in freezing tolerance have been reported (Loomis, 1985; Ansart & Vernon, 2003). In land snails, several species have been shown to exhibit freezing avoidance by lowering their supercooling points in winter (Riddle & Miller, 1988; Ansart & Vernon, 2003). In freshwater snails, for which little information is available, the pulmonate *Gyraulus acronicus* exhibits seasonal fluctuation in cold hardiness, developing a capability for freezing tolerance during winter (Olsson, 1984).

As an invasive freshwater snail, *P. canaliculata* has adopted various strategies for overwintering in paddy fields in temperate Japan. The snails bury themselves in the paddy soil or move under rice straw before the onset of winter. This behavior is apparently to avoid cold weather and desiccation. In fact, the environment under straw was much milder, with higher humidity, than that on the soil surface,

and that in the soil itself would be expected to be more favorable for overwintering than that under straw. In fact, snails that successfully buried themselves in the soil attained higher survivorship. In addition to this behavioral avoidance, this study clearly demonstrated physiological change in *P. canaliculata*, allowing it to develop cold hardiness in winter. This change possibly includes a decline in the supercooling temperature. However, other unknown physiological processes might also be involved, because *P. canaliculata* often died at temperatures above zero (see the mortalities after two days at 3°C in Fig. 1).

We suspect that *P. canaliculata* may not be fully adapted to its new habitat of temperate paddy fields in Japan. Many snails – about one third of those examined in this study – failed to bury themselves in the paddy soil, despite the fact that this would have ensured higher survivorship during winter. The situation is more serious if there is little straw in paddy fields (farmers often burn straw after the rice harvest), and no snails on the soil surface survived in such fields in Kumamoto (Kiyota & Sogawa, 1996). Even when the snails succeeded in burying themselves, the proportion that survived the winter was very low (7.8%) in this study. This suggests that the physiological enhancement developed by *P. canaliculata* before winter is insufficient. Undoubtedly, a cold winter imposes strong natural selection on the *P. canaliculata* population. We do not know whether the capability of developing cold hardiness before winter is an original ability of *P. canaliculata* in its native habitat, or a characteristic that has evolved after its invasion of temperate paddy fields. *Pomacea canaliculata* was introduced to many countries in tropical Asia where it does not encounter cold temperature stress. If we compare responses of the snails to temperature among populations in different regions, *P. canaliculata* may become a good model for studying the adaptive strategies of animals that have invaded a new habitat.

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